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DESCRIPTION**TITLE OF THE INVENTION**

FUNCTIONAL DOMAIN AND ASSOCIATED MOLECULE OF DOCK2 ESSENTIALLY
REQUIRED IN LYMPHOCYTE MIGRATION

Technical Field

The present invention relates to the identification of DOCK2 domain by using a deletion mutant, and a method for screening a substance interfering in the binding of DOCK 2 and SH3 domain of DOCK 2, particularly to a method for screening a substance interfering in the association of DOCK2 and ELMO, a method for screening a substance interfering in the association of ELMO and GDP/GTP exchange factor such as Tiam, or to a method for searching therapeutic agents for immune-related diseases, such as allergy, autoimmune diseases, GvH or graft rejection, with the use of these screening methods.

Background Art

Immune response is a regulatory mechanism indispensable against infection for a living body, and immune cells are patrolling constantly in the living body, to respond rapidly to various sources of infection. Such characteristics that constitutive cells are moving continuously are not recognized in other complex living systems, and have been developed specifically in the immune system. Among the immune cells, cells such as neutrophils, macrophages are known to function during primary defense of infection, while T- and B-lymphocytes trigger antigen-specific immune response by recognizing external foreign substances via the antigen receptor. The

above T- and B-lymphocytes differentiate in primary lymphoid organs such as thymus and bone marrow, and transfer to a particular compartment in second lymphoid organs such as spleen, lymph nodes, Payer's patch (lymphoid organs in the small intestine), and by recognizing antigens gathered there from various organs via the antigen receptor, induce specific immune response. At that time, the transfer of lymphocytes to a particular site of second lymphoid organ is very important for the formation of immune response. Heretofore, the transfer of the lymphocytes was known to be induced by protein called generally various chemokines, while the molecule mechanism that controls the mobility of the lymphocytes themselves remained unknown.

Change of cell polarization and cytoskeletal reorganization were indispensable for the cells movement (Cell 84, 359-369, 1996), and these were known to be controlled by G protein of low molecular weight such as Rho, Rac and Cdc42 (Proc. Natl. Acad. Sci. USA 92, 5027-5031, 1995; Science 279, 509-514, 1998; J. Cell Biol. 141, 1147-1157, 1998; Science 287, 1037-1040, 2000). Among these, Rac particularly provides driving force at the time of cell migration, by forming an actin-rich protrusion, called filopial protrusion (Science 279, 509-514, 1998; Cell 103, 227-238, 2000). On the other hand, molecules showing structural homology called CED5, DOCK 180 and Myoblast city (MBC) were identified in *Caenorhabditis elegans*, human and *Drosophila melanogaster*. These molecules are called CDM family molecules by their initials, and all of them are thought to be related to cytoskeletal reorganization by functioning upstream of Rac (Cell 84, 359-369, 1996; J. Cell Biol. 138, 589-603, 1997; Nature 392, 501-504, 1998; Genes Dev. 12,

3331-3336, 1998; Genes Dev. 12, 3337-3342, 1998; Nature Cell Biol. 2, 131-136, 2000). Although genetic analysis with the use of a deletion mutant has shown that the above CED-5 and Myoblast City are crucial for cell migration of particular types of cells, (J. Cell Biol. 138, 589-603, 1997; Nature 392, 501-504, 1998; Nature Cell Biol. 2, 131-136, 2000), physiological relevance of the CDM family proteins in mammals remained unknown.

It is known that DOCK2 (KIAA0209; DNA Res.3, 321-329) encodes another member of the CDM family proteins, which is specifically expressed in human haematopoietic cells, and that the DOCK2 binds to activate Rac in 293T kidney cells (Biochem, Biophys. Acta 1452, 179-187, 1999). On the other hand, the present inventors isolated a new gene Hch belonging to the CDM family from mouse thymus cDNA library, and found that the gene product comprises 1828 amino acids, and encodes SH3 domain at the N terminus (Nature, 412, 826-831, 2001). Moreover, the present inventors confirmed by Northern Blot analysis using mouse organs that whereas DOCK180 was expressed in various organs, the expression of Hch was restricted to thymus and spleen. Further, by an analysis using cell lines they confirmed that Hch expression was observed in all T-, B- and macrophage cells, with the exception of two mutant T-cell lines. Furthermore, it has been revealed that a significant change in cell morphology and enhancement of adhesion were observed by introducing Hch into mutant T-cell line lacking Hch expression. Though 1677 of the 1828 amino acids encoded by Hch are identical to human DOCK2, and Hch was thought to be mouse DOCK2 homologue, the physiological function remained unknown.

The present inventors identified DOCK2 as a molecule belonging to the CDM family, expressing specifically in lymphocytes as mentioned above, and by generating the knockout mice, they revealed that the molecule was indispensable to lymphocyte migration (Nature, 412, 826-831, 2001). In DOCK2-deleted lymphocytes, active Rac is not detected by any of chemokine stimulation. Therefore, it can be thought that DOCK2 regulates lymphocyte migration via Rac activation. However, it remains unknown by which mechanism DOCK2 activates Rac. Rac functions as a molecule switch, and is activated by a GDP/GTP exchange factor (GEF). Though DOCK2 binds with Rac, it is hard from its structure, to think that it functions as GEF. Therefore, it is estimated that DOCK2 activates Rac by recruiting GEF via other molecules.

Recently, CED-12 being a molecule that associates with CED-5, which is one of the CDM family molecules, and that regulates cytoskeleton has been identified in *C. elegans*, and ELMO-1, -2 and -3 were reported as their mammalian homologues (Cell, 107, 27-41, 2001). Moreover, several dozens of GDP/GTP exchange factors (GEF) were known heretofore, and among these GEFs, as a molecule functioning as Rac-specific GEF, the following are known: Tiam-1 and -2 that determines the invasion to thymoma cell lines (Cell, 77, 537-549, 1994; Nature, 375, 338-340, 1995); Vav1 that regulates T cell receptor signal (Nature, 385, 169-172, 1997) besides Vav2, Vav3; Trio (J. Cell Science, 113, 729-739, 2000); STEF (J. Biol. Chem., 277, 2860-2868, 2002); and P-Rex1 (Cell, 108, 809-821, 2002). All these five molecules have a common domain, and comprise a function to provide GTP to Rac.

Autoimmune diseases and graft rejection are caused by the

invasion of lymphocytes into the target organ. Therefore, it is thought that DOCK2 might be a suitable target molecule to treat or prevent such diseases or pathology. The object of the present invention is to identify the functional domain of DOCK2 by using a deletion mutant, to screen a substance interfering in the binding of DOCK2 and SH3 domain of DOCK2, particularly to provide a method for screening a substance interfering in the association of DOCK2 and ELMO, a method for screening a substance interfering in the association of ELMO and GDP/GTP exchange factor such as Tiam, or a method for searching therapeutic agents for immune-related diseases, such as allergy, autoimmune diseases, GvH or graft rejection with the use of these screening methods, and the like.

DOCK2 is a molecule expressed specifically in lymphocytes, comprised of 1828 amino acid residues including SH3 domain, that activates Rac and regulates cytoskeleton to determine lymphocyte mobility. The present inventors have made a keen study to solve the above object, found that Rac-activating ability was significantly decreased in DOCK2 mutant lacking 504 amino acid residues in the N terminus including SH3 domain of DOCK 2, and that actin polymerization could not be induced, and they identified ELMO1 as a molecule binding to this domain. Moreover, as the binding of DOCK2 and ELMO1 was completely inhibited by the single amino acid mutation of SH3 domain, they have found that DOCK2 associates with ELMO1 via SH3 domain. Furthermore, they have found that ELMO1 binds with Tiam1 functioning as Rac-specific GDP/GTP exchange factor (GEF). In other words, they have found that DOCK2 activates Rac by recruiting Tiam1 via ELMO1. Therefore, they found that by inhibiting intermolecular interaction of SH3 domain of DOCK2,

ELMO1 and Tiam1, the artificial control of lymphocyte migration was possible. The present invention has been thus completed with this knowledge.

Disclosure of the Invention

In other words, the present invention relates to a method for screening a substance interfering in the association of DOCK2 and ELMO, comprising the steps of contacting DOCK2, ELMO and a test substance, and then estimating the level of formation of association of DOCK2 and ELMO ("1"); a method for screening a substance interfering in the association of DOCK2 and ELMO, comprising the steps of contacting SH3 domain of DOCK2, ELMO and a test substance, and then estimating the level of formation of association of SH3 domain of DOCK2 and ELMO ("2"); a method for screening a substance interfering in the association of DOCK2 and C terminus domain of ELMO, comprising the steps of contacting DOCK2, C terminus domain of ELMO and a test substance, and then estimating the level of formation of association of DOCK2 and C terminus domain of ELMO ("3"); a method for screening a substance interfering in the association of DOCK2 and ELMO, comprising the steps of contacting SH3 domain of DOCK2, C terminus domain of ELMO and a test substance, and then estimating the level of formation of association of SH3 domain of DOCK2 and C terminus domain of ELMO ("4"); the method for screening a substance interfering in the association of DOCK2 and ELMO according to any one of "1" to "4", wherein DOCK2 or its SH3 domain and/or ELMO or its C-terminus domain is bound with a marker protein and/or peptide tag ("5"); the method for screening a substance interfering in the association of DOCK2 and ELMO according to anyone of "1" to "5", wherein an antibody

against ELMO or its C terminus domain is acted to DOCK2 or its SH3 domain fractionated by an antibody against DOCK2 or its SH3 domain, or an antibody against other peptides fused with DOCK2 or its SH3 domain, and the level of formation of association is estimated ("6"); the method for screening a substance interfering in the association of DOCK2 and ELMO according to any one of "1" to "6", wherein the level of formation of association is estimated by detecting GTP-binding form of activated-Rac ("7"); the method for screening a substance interfering in the association of DOCK2 and ELMO according to any one of "1" to "7", wherein the substance interfering in the association of DOCK2 and ELMO is a substance promoting or suppressing the function of regulating lymphocyte migration ("8"); the method for screening a substance interfering in the association of DOCK2 and ELMO according to any one of "1" to "7", wherein the substance interfering in the association of DOCK2 and ELMO is a substance inhibiting the binding of DOCK2 and ELMO ("9"); the method for screening a substance interfering in the association of DOCK2 and ELMO according to any one of "1" to "9", wherein ELMO is ELMO1 ("10"); a method for searching a therapeutic agent for immune related diseases such as allergy, autoimmune diseases, GvH, and graft rejection wherein the method for screening a substance interfering in the association of DOCK2 and ELMO according to any one of "1" to "10" is used ("11"); and a method for searching a therapeutic agent for diseases caused by the suppression of lymphocyte migration, which promotes cytoskeletal reorganization by activating Rac, wherein the method for screening a substance interfering in the association of DOCK2 and ELMO according to any one of "1" to "10" is used ("12").

Moreover, the present invention is related to a method for screening a substance interfering in the association of ELMO and GDP/GTP exchange factor, comprising the steps of contacting ELMO, GDP/GTP exchange factor and a test substance, and then estimating the level of formation of association of ELMO and GDP/GTP exchange factor ("13"); a method for screening a substance interfering in the association of ELMO and GDP/GTP exchange factor, comprising the steps of contacting N terminus domain of ELMO, GDP/GTP exchange factor and a test substance, and then estimating the level of formation of association of N terminus domain of ELMO and GDP/GTP exchange factor ("14"); the method for screening a substance interfering in the association of ELMO and GDP/GTP exchange factor according to "13" or "14", wherein ELMO or its N terminus domain and/or GDP/GTP exchange factor is fused with another peptide ("15"); the method for screening a substance interfering in the association of ELMO and GDP/GTP exchange factor according to any one of "13" to "15", wherein an antibody against ELMO or its N terminus domain is acted to a GDP/GTP exchange factor fractionated by an antibody against GDP/GTP exchange factor or by an antibody against another peptide fused with GDP/GTP exchange factor, and the level of formation of association is estimated ("16"); the method for screening a substance interfering in the association of ELMO and GDP/GTP exchange factor according to any one of "13" to "16", wherein the level of formation of association is estimated by detecting GTP-binding form of activated-Rac ("17"); the method for screening a substance interfering in the association of ELMO and GDP/GTP exchange factor according to any one of "13" to "17", wherein the substance interfering in the association of ELMO

and GDP/GTP exchange factor is a substance promoting or suppressing the function of regulating lymphocyte migration ("18"); the method for screening a substance interfering in the association of ELMO and GDP/GTP exchange factor according to any one of "13" to "17", wherein the substance interfering in the association of ELMO and GDP/GTP exchange factor is a substance inhibiting the binding of ELMO and GDP/GTP exchange factor ("19"); the method for screening a substance interfering in the association of ELMO and GDP/GTP exchange factor according to any one of "13" to "19", wherein ELMO is an ELMO bound with DOCK2 ("20"); the method for screening a substance interfering in the association of ELMO and GDP/GTP exchange factor according to any one of "13" to "20", wherein ELMO is ELMO1 ("21"); the method for screening a substance interfering in the association of ELMO and GDP/GTP exchange factor according to any one of "13" to "21", wherein the GDP/GTP exchange factor is a Rac-specific GDP/GTP exchange factor ("22"); the method for screening a substance interfering in the association of ELMO and GDP/GTP exchange factor according to "22", wherein the Rac-specific GDP/GTP exchange factor is Tiam1 ("23"); a method for searching a therapeutic agent for immune related diseases such as allergy, autoimmune diseases, GvH, and graft rejection, wherein the method for screening a substance interfering in the association of ELMO and GDP/GTP exchange factor according to any one of "13" to "23" is used ("24"); and a method for searching a therapeutic agent for diseases caused by the suppression of lymphocyte migration, which promotes cytoskeletal reorganization by activating Rac, wherein the method for screening a substance interfering in the association of ELMO and GDP/GTP exchange factor according to any one of "13" to "23" is used ("25").

Furthermore, the present invention relates to a method for screening a substance for promoting or suppressing Rac activation, comprising the steps of contacting DOCK2, ELMO, GDP/GTP exchange factor and a test substance, and then estimating the level of formation of association of DOCK2 and ELMO, or the level of formation of association of ELMO and GDP/GTP exchange factor ("26"); a method for screening a substance for promoting or suppressing Rac activation, comprising the steps of contacting SH3 domain of DOCK2, ELMO, GDP/GTP exchange factor and a test substance and then estimating the level of formation of association of SH3 domain of DOCK2 and ELMO, or the level of formation of association of ELMO and GDP/GTP exchange factor ("27"); the method for screening a substance for promoting or suppressing Rac activation according to "26" or "27", wherein the level of formation of association is estimated by detecting GTP-binding form of activated-Rac ("28"); the method for screening a substance for promoting or suppressing Rac activation according to any one of "26" to "28", wherein ELMO is an ELMO bound with DOCK2 ("29"); the method for screening a substance for promoting or suppressing Rac activation according to any one of "26" to "29", wherein ELMO is ELMO1 ("30"); the method for screening a substance for promoting or suppressing Rac activation according to any one of "26" to "30", wherein the GDP/GTP exchange factor is a Rac-specific GDP/GTP exchange factor ("31"); the method for screening a substance for promoting or suppressing Rac activation according to "31", wherein the Rac-specific GDP/GTP exchange factor is Tiam1 ("32"); a method for searching a substance for promoting or suppressing the function of regulating lymphocyte migration, wherein the method for

screening a substance promoting or suppressing Rac activation according to any one of "26" to "32" is used ("33"); a method for searching a therapeutic agent for immune related diseases such as allergy, autoimmune diseases, GvH, and graft rejection, wherein the method for screening a substance for promoting or suppressing Rac activation according to any one of "26" to "32" is used ("34"); and a method for searching a therapeutic agent for diseases caused by the suppression of lymphocyte migration, which promotes reconstruction of cytoskeleton by activating Rac, wherein the method for screening a substance for promoting or suppressing Rac activation according to any one of "26" to "32" is used ("35"); a therapeutic agent for immune related diseases such as allergy, autoimmune diseases, GvH and graft rejection, obtained by the searching method according to "11", "24" or "34" ("36"); a therapeutic agent for diseases caused by the suppression of lymphocyte migration, promoting cytoskeletal reorganization by activating Rac, obtained by the searching method according to "12", "25" or "35" ("37"); a method for screening a substance inhibiting DOCK2-function, by targeting N terminus domain of DOCK2 including SH3 domain, comprising the steps of contacting SH3 domain of DOCK2, the SH3 domain-binding protein and a test substance, and then estimating the level of formation of association of DOCK2 and SH3 domain-binding protein ("38"); and a method for screening a substance inhibiting DOCK2-function, by using a transgenic cell line expressing full-length DOCK2 and DOCK2-deleted mutants, comprising the steps of measuring and estimating the level of Rac activation in these cell lines, identifying the functional domain of DOCK2, searching a molecule associated with functional domain that associates with the functional domain,

contacting the functional domain of DOCK2, the molecule associated with functional domain and a test substance, and estimating the level of formation of association of functional domain of DOCK2 and molecule associated with functional domain of DOCK2 ("39").

Brief Description of Drawings

Fig. 1 is a figure showing that DOCK2 binds with ELMO1 at its N terminus domain.

A is a view showing a frame format of the structure of DOCK2 and DOCK2-deleted mutants. In the figure, the black-colored part is the SH3 domain.

B is a figure showing the analysis of the binding with ELMO1 by immunoprecipitation and Western Blot method, by transfecting genes encoding DOCK2 or DOCK2-deleted mutants to 293T cells with PcdNA ELMO1-V5 and by collecting the cells 48 hours later. Types of samples used for analysis, antibodies used for immunoprecipitation and Western Blot are shown on the left side.

Fig. 2 is a set of pictures showing that the Rac-activating ability is significantly decreased and that actin polymerization cannot be induced in DOCK2 Δ N lacking N terminus domain essential for the binding with ELMO1.

A is a picture showing the analysis of the expression of DOCK2 or DOCK2 Δ N in BE α 16-3, N3-5, and transgenic cell lines (17-11, 84-3) by Western Blot with the use of polyclonal antibody against DOCK2. In the figures, NS means non-specific band.

B is a picture that activated Rac is detected by pulling-down cell extract of 84-3, 17-11, BE α 16-3 with GST fusion protein of PAK1 Rac-binding domain, and by staining with anti-Rac antibody.

C is a picture showing the investigation of cell polarization and actin polymerization by staining BEα16-3, 17-11, 84-3 with propidium iodide and phalloidin.

Fig.3 is a picture showing that DOCK 2 associates with ELMO1 via its SH3 domain.

A is a figure showing the amino acid sequence 10-89 including DOCK2 SH3 domain. Amino acid residues substituted to glutamic acid are shown in bold letter.

B is a figure showing the analysis of the binding of DOCK2 with ELMO1 by immunoprecipitation and Western Blot method, by transfecting genes encoding DOCK2 or DOCK2 SH3-deleted mutants to 293T cells with PcdNA ELMO1-V5 and by collecting the cells 48 hours later. Types of samples used for analysis, antibodies used for immunoprecipitation and Western Blot are shown on the left side.

Fig.4 is a figure showing that ELMO1 is bound with DOCK2 at its C terminus domain.

A is a view showing a frame format of the structure of ELMO1 and of ELMO1-deleted mutants used in this experiment.

B is a figure showing the analysis of the binding of ELMO1 with DOCK2 by immunoprecipitation and Western Blot method, by transfecting genes encoding ELMO1 or ELMO1-deleted mutants to 293T cells with PcdNA DOCK2-HA or a control vector and by collecting the cells 48 hours later. Types of samples used for analysis, antibodies used for immunoprecipitation and Western Blot are shown on the left side.

Fig. 5 is a figure showing that ELMO1 is bound to Tiam1 at its N terminus domain.

A is a view showing a frame format of the structure of ELMO1 and of ELMO1-deleted mutants used in this experiment.

B is a figure showing the analysis of the binding with Tiam1 by immunoprecipitation and Western Blot method, by transfecting genes encoding ELMO1 or ELMO1-deleted mutants to 293T cells with PCI Tiam1-HA or a control vector and by collecting the cells 48 hours later. Types of samples used for analysis, antibodies used for immunoprecipitation and Western Blot are shown on the left side.

Fig. 6 is a schematical view of the Rac-activating mechanism by DOCK2.

It is a figure showing that DOCK2 activates Rac via ELMO1 by recruiting Tiam1 functioning as GEF of Rac.

Best Mode of Carrying Out the Invention

As for the method for screening a substance interfering in the association of DOCK2 and ELMO of the present invention, there is no specific limitation as long as it is a method comprising the steps of contacting DOCK2, ELMO and a test substance, and then estimating the level of formation of the association of DOCK2 and ELMO; a method comprising the steps of contacting SH3 domain of DOCK2, ELMO and a test substance, and then estimating the level of formation of the association of SH3 domain of DOCK2 and ELMO; a method comprising the steps of contacting DOCK2, C terminus domain of ELMO and a test substance, and then estimating the level of formation of the association of DOCK2, C terminus domain of ELMO; a method comprising the steps of contacting SH3 domain of DOCK2, C terminus domain of ELMO, and a test substance, and then estimating the level of formation of the association of SH3 domain of DOCK2, C terminus of ELMO. Moreover, as for the above-mentioned DOCK2 or its SH3 domain and/or ELMO or its C

terminus domain, a fusion protein or a fusion peptide wherein these and marker protein and/or peptide tag are bound can be used. Moreover, as for the above ELMO, ELMO1, ELMO2, ELMO3 can be specifically exemplified, and ELMO1 can be preferably exemplified.

As for the above SH3 domain of DOCK2, a DOCK2 mutant having a function to associate with ELMO, and that is a peptide containing a whole or a part of SH3 domain of DOCK2 can be exemplified, and specific examples include DOCK2N comprising amino acid residue 1-502 of DOCK2 and DOCK2 Δ C comprising amino acid residue 1-1311 of DOCK2. Furthermore, as for the above C terminus domain of ELMO, a mutant of ELMO having the function to associate with SH3 domain of DOCK2, and that is a peptide containing a whole or a part of C terminus domain of ELMO can be exemplified, and specific examples include ELMO1-del1 comprising amino acid residue 147-727 of ELMO1, and ELMO1-del8 comprising amino acid residue 345-727 of ELMO1. Hereinafter, DOCK2 and the above SH3 domain of DOCK2 can be referred together to as "DOCK2 and the like", and ELMO such as ELMO1 and the above C terminus domain of ELMO can be referred together to as "ELMO and the like".

The above DOCK2 mutant or ELMO mutant can be prepared by modifying DOCK2 genes or ELMO genes according to a common procedure. As for DOCK2 genes, Hch (mouse DOCK2) genes (GenBank Accession No. AY027438; Nature, Vol 412, 23 August, 826-831, 2001) and human DOCK2 genes (XM_047961; DNA Res. 3, 321-329) can be specifically exemplified, but the origin of DOCK2 genes is not limited to mouse, human and the like. Moreover, as for ELMO genes such as ELMO1, besides mouse ELMO1 genes (AF398883; Cell, Vol. 107 (1), 27-41, 2001) and human ELMO1 genes

(AF398885; Cell, Vol. 107(1) 27-41, 2001), ELMO2 genes (human AF398886, mouse AF398884), ELMO3 genes (human NM_024712) can be specifically exemplified. However, the origin of DOCK2 and ELMO genes is not limited to mouse, human and the like. Additionally, the amino acid sequence of mouse DOCK2, human DOCK2, mouse ELMO1, and human ELMO1 are shown as Seq. ID Nos. 1, 2, 3 and 4, respectively.

As for a marker protein in a fusion protein or fusion peptide wherein the above DOCK2 and the like or ELMO and the like are bound with a marker protein and/or peptide tag, there is no specific limitation as long it is a marker protein conventionally known, and alkaline phosphatase, Fc domain of an antibody, HRP, and GFP can be exemplified. Moreover, as for a peptide tag, examples include peptide tags conventionally known, including epitope tags such as HA, FLAG and Myc; affinity tag such as GST, maltose-binding protein, biotinylated peptide and oligo-histidine. The fusion protein or fusion peptide can be prepared by a common procedure, and can separate/fractionate fusion protein or fusion peptide with DOCK2 and the like, ELMO1 and the like and HA-tag, by using specific antibody against HA tag.

In the method for screening a substance interfering in the association of DOCK2 and ELMO such as ELMO1, as for a method for contacting DOCK2 and the like, ELMO and the like, and a test substance, there is no specific limitation as long as it is a contacting method that can evaluate the level of the formation of the association of DOCK2 and the like and ELMO and the like, and examples include a method for contacting DOCK2 and the like and ELMO and the like, in the presence of a test substance in a cell-free system; a method for introducing an expression

vector integrated with ELMO and the like or genes encoding ELMO and the like, in a cell expressing DOCK2 and the like together with a test substance; a method for introducing an expression vector integrated with DOCK2 and the like or genes encoding DOCK2 and the like, in a cell expressing ELMO and the like together with a test substance; or a method for introducing an expression vector integrated with DOCK2 and the like or genes encoding DOCK2 and the like, an expression vector integrated with EOMO and the like or genes encoding ELMO and the like, and a test substance, in a cell not expressing DOCK2 and the like nor ELMO and the like.

As for cells used for contacting with the above test substance, bacterial prokaryotic cells such as E. Coli, streptomyces, Bacillus subtilis, Streptococcus and Staphylococcus; eukaryotic cells such as yeast and Aspergillus; insect cells such as Drosophila S2 and Spodoptera Sf9; plant and animal cells such as L cells, CHO cells, COS cells, HeLa cells, C127 cells and BALB/c3T3 cells (including mutant strain lacking dihydrofolate reductase or thymidine kinase), BHK21 cells, HEK293 cells, Bowes melanoma cells and oocytes can be exemplified, and animal cells are preferable. Moreover, as for the method for introducing DOCK2 and the like or ELMO and the like in these cells, besides the above methods for introducing genes, a noncytotoxic reagent such as Chariot (Active Motif) that can form a non-covalent binding with an enormous molecule, change the structure of an enormous molecule such as protein, and that can deliver the enormous molecule such as protein into the cells, can be used.

As for the above expression vector, expression vector for animal cells are preferable, and examples of the expression

vector for animal cells include: expression system derived from chromosome, episome, and virus; for example vectors derived from bacterial plasmid, yeast plasmid, papovavirus such as SV40, vaccinia virus, adenovirus, fowl poxvirus, pseudorabies virus, lentivirus, and retrovirus; vectors derived from bacteriophage, transposon, or from combination thereof, for example those derived from plasmid and bacteriophage elements, such as cosmids and phagemids. These expression systems can include regulatory sequences that not only induce expression but also regulate expression. Moreover, liposome can be used in place of expression vectors for animal cells. Further, the introduction of the expression vectors for animal cells into cells can be performed by a method described in various standard laboratory manuals such as Davis et al. (BASIC METHODS IN MOLECULAR BIOLOGY, 1986) and Sambrook et al. (MOLECULAR CLONING: A LABORATORY MANUAL, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989), and examples include calcium phosphate transfection, DEAE-dextran mediated transfection, transvection, microinjection, cationic lipid mediated transfection, electroporation, transduction, scrape loading, ballistic introduction, and infection.

In the method for screening a substance interfering the association of DOCK2 and ELMO such as ELMO1 of the present invention, as for the method for estimating the level of formation of the association of DOCK2 and the like and ELMO and the like, a method for measuring/estimating immunochemically the level of formation of the association of DOCK2 and the like and ELMO and the like, by acting an antibody against ELMO and the like to DOCK2 and the like being separated/fractionated, or by acting an antibody against DOCK2 and the like to ELMO and

the like being separated/fractionated, can be exemplified. To separate/fractionate DOCK2 and the like or ELMO and the like, specific antibodies against DOCK2 and the like or ELMO and the like or tag-specific antibodies can be used. Moreover, yeast two hybrid system that can detect protein-protein interaction by using a minute amount of protein and without labeling; or a biosensor using the surface plasmon resonance reaction that can observe at real time as a surface plasmon resonance signal; or a method for measuring/estimating the level of formation of the association by using NMR method that can detect the change of tertiary structure, can be also exemplified. Moreover, publicly known methods for searching interacting protein, such as far western method using E. coli expression system and a method using affinity chromatography can be exemplified.

In the method for screening a substance interfering in the association of DOCK2 and ELMO of the present invention, as another method to estimate the level of formation of the association of DOCK2 and the like and ELMO and the like, an estimation method by detecting a GTP-binding form of activated Rac can be exemplified. To detect activated Rac, a pull-down method using GST fusion protein of PAK1 Rac-binding domain can be used.

As for samples to be tested in the method for screening a substance interfering in the association of DOCK2 and ELMO of the present invention, for example, peptides, proteins, synthesized compounds, microbial fermented materials, marine organism extracts, plant extracts, prokaryotic cells extract, eukaryotic unicellular extract, animal cells extract or library thereof can be exemplified. Furthermore, in the method for

screening a substance interfering in the association of DOCK2 and ELMO of the present invention, control experiment can be carried out simultaneously. As for control, negative control that does not affect the formation of association of DOCK2 and the like and ELMO and the like, and/or positive control that affect the formation of association of DOCK2 and the like and ELMO and the like can be used.

As for the above substances interfering in the association of DOCK2 and ELMO, substances promoting or suppressing the function of regulating lymphocyte migration, particularly a substance suppressing the function of regulating lymphocyte migration such as substances inhibiting the binding of DOCK2 and ELMO. As for the function of regulating lymphocyte migration, there is no specific limitation as long as it is a function regulating the mobility of lymphocytes based on the expression of DOCK2 genes. Examples include a function promoting cytoskeletal reorganization, in particular actin polymerization in lymphocytes by activating Rac and making a Rac-GTP binding; a function of migrating lymphocytes in response to stimulation of chemokines such as SLC, SDF-1, and BLC; homing function to a secondary lymphoid organ such as spleen, lymph nodes, payer's notch and the like; function of transferring mature thymus T cells to peripheral blood in response to ELC chemokine stimulation; or a function of migrating CD4⁺CD8⁺ immature thymus cells in response to SDF-1 chemokine stimulation.

The present invention relates also to a method for screening a substance interfering in the association of ELMO and GDP/GTP exchange factor (GEF), or to a method for screening a substance promoting or suppressing Rac activation. As for

the method for screening a substance interfering in the association of ELMO and GEF, there is no specific limitation as long as it is a method comprising the steps of contacting ELMO, GEF, and a test substance, and then estimating the level of formation of association of ELMO and GEF; or a method comprising the steps of contacting N terminus domain of ELMO, GEF and a test substance, and then estimating the level of formation of association of N terminus domain of ELMO and GEF. Moreover, as for the method for screening a substance promoting or suppressing Rac activation, there is no specific limitation as long as it is a method comprising the steps of contacting DOCK2, ELMO, GEF and a test substance, or by contacting SH3 domain of DOCK2, ELMO, GEF and a test substance, and then estimating the level of formation of association of DOCK2 and ELMO, or the level of formation of association of ELMO and GEF. Further, as for the above ELMO, ELMO bound with DOCK2 can be used.

As for the above ELMO, examples include ELMO1, ELMO2, ELMO3, and among these, ELMO1 can be preferably exemplified. Moreover, as for the above GEF, Rac-specific GDP/GTP exchange factors such as Tiam1, Tiam2, Vav1, Vav2, Vav3, Trio, STEF, P-Rex1 are preferable, and among these, Tiam1 can be preferably exemplified. As for the above Tiam1 gene, mouse Tiam1 gene (NM_009384; Cell Vol. 77(4), 537-549, 1994), human Tiam1 gene (NM_003253; Oncogene Vol. 10(7), 1371-1376, 1995) can be exemplified, but the origin of Tiam1 gene is not limited to mouse, human and the like. Amino acid sequences of mouse Tiam1, human Tiam1 are shown in Seq. ID. Nos. 5 and 6, respectively.

Methods used for the above method for screening a substance interfering in the association of DOCK2 and ELMO,

including the above method for screening a substance interfering in the association of ELMO and GEF, or a method for estimating the level of formation of association of ELMO and GEF, a method for estimating the level of formation of association of DOCK2 and ELMO, a method of using ELMO fused with other peptides, or its N terminus, and GEF, in the method for screening a substance for promoting or suppressing Rac activity, can be applied accordingly.

By using the method for screening a substance interfering in the association of DOCK2, ELMO such as ELMO1, the method for screening a substance interfering in the association of ELMO and GEF, the method for screening a substance promoting or suppressing Rac activation of the present invention, particularly the method for screening a substance promoting or suppressing the function of regulating lymphocyte migration, screening of preventive/therapeutic agents of immune related diseases such as allergy, autoimmune diseases, GvH, graft rejection targeting DOCK2 can be possible. As it can be anticipated that substances suppressing the function of regulating lymphocyte migration obtained by the method for screening a substance promoting or suppressing the function of regulating lymphocyte migration, such as anti-DOCK2 SH3 domain antibody, DOCK2 SH3 domain-binding molecule (including low molecular compounds), antisense strand of DOCK2 gene, antibodies recognizing specifically the DOCK2 SH3 domain-binding site of C terminus domain of ELMO such as ELMO1, molecules binding to the DOCK2 SH2 domain-binding site of C terminus domain of ELMO such as ELMO1 (including low molecular compounds), antibodies recognizing specifically GEF-binding site such as Tiam1 of N terminus domain of ELMO such as ELMO1,

molecules binding to GEF-binding site such as Tiam1 of N terminus domain of ELMO such as ELMO1 (including low molecular compound), or antisense strand of ELMO such as ELMO1, can suppress artificially lymphocyte mobility, the possibility for these suppressive substances to be a therapeutic agent against immune-related diseases such as allergy, autoimmune diseases, GvH, graft rejection is high. When the therapeutic agent is used as drugs, various prescribed compounds such as pharmaceutically acceptable normal carrier, bonding agent, stabilizing agent, excipient, diluent, pH buffer agent, disintegrator, solubilizer, dissolving adjuvant, isotonic agent can be added, and can be administered by an administration form used generally, for example orally in formulation form such as powder, granule, capsule, syrup, and suspending agent, or parenterally in form of injection those formulated in form of solution, emulsion, suspending solution and the like.

Moreover, when using the method for screening a substance interfering in the association of DOCK2 and ELMO1, the method for screening a substance interfering in the association of ELMO1 and Tiam1, the method for screening a substance promoting or suppressing Rac activity of the present invention, in particular the method for screening a substance promoting the function of regulating lymphocyte migration, cytoskeletal reorganization is promoted by activating Rac, and thus, screening of preventive/therapeutic agents against diseases caused by suppression of lymphocyte migration, such as various cancers, or immunodeficiency caused by drugs/irradiation, can be possible.

Furthermore, as for the method for screening a substance inhibiting DOCK2 function of the present invention, examples

include a method making the N terminus domain of DOCK2 including SH3 domain as target, comprising the steps of contacting SH3 domain of DOCK2 and the SH3 domain-binding protein and a test substance, and then estimating the level of formation of association of DOCK2 and SH3 domain-binding protein; and a method by using transgenic cell line expressing full length DOCK2 and DOCK2-deleted mutant, measuring/estimating the level of Rac activation in these cell lines, identifying the functional domain of DOCK2, searching a molecule associated with functional domain that associates with the functional domain, contacting the functional domain of DOCK2, the molecule associated with functional domain and a test substance, and estimating the level of formation of association of functional domain of DOCK2 and molecule associated with the functional domain. As for the method for contacting with a test substance, the method for estimating the level of formation of association, or the method for measuring the level of Rac activation, the methods mentioned above can be used. As for the method for identifying the functional domain of DOCK2, or for the preparation of transgenic cell line expressing full length DOCK2 and DOCK2-deleted mutant, methods described in the following examples can be used.

In the following, the present invention will be explained in detail by reference to the examples, while the technical scope of the present invention is not limited to these examples.

Example 1 (Binding of N terminus domain of DOCK2 and ELMO 1)

Recently, CED-12 has been identified as a molecule that associates with CED-5 and regulates cytoskeleton in nematodes, and ELMO1 has been reported as its mammal homologue (Cell 107,

27-41, 2001). Therefore, in order to investigate whether DOCK2 binds with ELMO1 or not, by using PcdNA/His max vector (Invitrogen), gene constructs encoding full length DOCK2 or various DOCK2-deleted mutants in which HA tag (YPYDVPDYA: Seq. ID No. 7) is introduced at the C terminus (PcdNA DOCK2-HA, PcdNA DOCK2 N-HA, PcdNA DOCK2 Δ C-HA, PcdNA DOCK2 Δ N-HA), were constructed. Then, the gene constructs were introduced into 293T cells (provided by Dr. Shinji Hatakeyama, Kyushu University) together with a gene in which ELMO1 cDNA is introduced into PcdNA V5-His vector (Invitrogen) (PcdNA ELMO1-V5). DOCK2 construct was prepared from genes isolated by the present inventors (Nature, 412, 826-831, 2001), and ELMO1 construct was prepared from mouse tissue cDNA by PCR according to a common method. The genes encoding the used DOCK2-deleted mutant are as follows, and they are shown schematically in Fig. 1.

- 1) PcdNA DOCK2 N-HA; genes encoding amino acid residue 1 - 502 of DOCK2
- 2) PcdNA DOCK2 Δ C-HA; genes encoding amino acid residue 1-1311 of DOCK2
- 3) PcdNA DOCK2 Δ N-HA; genes encoding amino acid residue 505-1828 of DOCK2

The cells were collected 48 hours after gene introduction, dissolved with Lysis buffer (Cell signaling), and analysed by Western Blot method using anti-V5 antibody (Invistrogen) to immunoprecipitants by total cell lysate and anti-HA antibody (Roche). For each of total cell lysate, a band of approximately 100-KD corresponding to ELMO1 was detected for anti-V5 antibody (Fig. 1B; top). However, for the immunoprecipitants, a band

corresponding to ELMO1 was detected, when genes encoding full length DOCK2, DOCK2 Δ C and DOCK2 N, while no band was detected when DOCK2 Δ N lacking amino acid residues from N terminus to 504 of DOCK2 (Fig. 1B; lower figure of the middle line). From these results, it has been clarified that DOCK2 associates with ELMO1 in the domain of amino acid residues from its N terminus to 502.

Example 2 (Rac activation in DOCK2 Δ N lacking the N terminus domain)

To clarify the influence of the association with ELMO1 to the function of DOCK 2, gene constructs encoding full length DOCK2 and a mutant lacking 504 amino acid residues of the N terminus of DOCK2 (DOCK2 Δ N) were constructed by using PBJ1 vector. Then, a stable transgenic cell strain was established by introducing the gene constructs into the T cell strain, BE α 16-3 (provided from National Jewish Center, Dr. Philppa Marrack), wherein the expression of DOCK 2 gene is deleted. N3-5 is a wild-type T cell strain expressing DOCK2, and 17-11 (Nature, 412, 826-831, 2001) and 84-3 are transgenic cell strains expressing full length DOCK2 and DOCK2 Δ N, respectively, that the present inventors have established. In the Western Blot analysis using anti-DOCK2 polyclonal antibody that the present inventors have prepared, the expression of DOCK2 and DOCK2 Δ N was approximately the same in 17-11 and 84-3 (Fig. 2A). Therefore, by targeting to 17-11 and 84-3, Rac activity in these cell strains was compared and analyzed by pull-down method using GST fusion protein of PAK1 Rac binding domain. In 17-11 expressing full length DOCK2, GTP-binding form of activated Rac was easily detected, whereas in 84-3 expressing DOCK2 Δ N lacking the binding site with ELMO 1, Rac activating ability was

significantly decreased (Fig. 2B). From the nuclear stain of 17-11 and 84-3 with PI (propidium iodide), it has been revealed that in any case, the nucleus is eccentrically located, in other words, that cell polarization is performed, which is different from BE α 16-3, the parent cell strain (Fig.2C; top). On the contrary, when these cells are stained with phalloidin, which is a probe for F-actin, actin polymerization was observed only for 17-11, and not in 84-3, as in the case of BE α 16-3, wherein the DOCK2 expression is deleted (Fig. 2C; bottom). From these results, the association of DOCK2 and ELMO1 has been suggested to be extremely crucial to the full activation of Rac as well as to cytoskeletal reorganization, relating thereof. From the above, it has been clarified that in DOCK2 Δ N, lacking N terminus domain being essential for the binding with ELMO1, the Rac-activating ability is significantly decreased, and that actin polymerization cannot be induced.

Example 3 (Association with ELMO1, via SH3 domain of DOCK2)

SH (Src-homolgy)3 domain known to be related with the protein-protein interaction is encoded at the N-terminus of DOCK2. As it was found that 502 amino acid residues at the N terminus of DOCK2 are crucial for the association with ELMO1, it was investigated if it is mediated by SH3 domain. Amino acid residues commonly conserved exist in the SH3 domain. Therefore, gene constructs encoding various DOCK2 SH3 mutants wherein HA tag is introduced into C terminus by using PcDNA/His max vector, were constructed. Then, these were introduced into 293T cells with PcDNA ELMO1-V5, and were analyzed in the same manner as in Fig.1B. Genes encoding DOCK2 SH3 mutant are as follows:

- 1) PcDNA L27E-HA; gene encoding mutant wherein leucine at the

- 27 position of DOCK2 is substituted to glutamic acid
- 2) PcdNA G32E-HA; gene encoding mutant wherein glycine at the 32 position of DOCK2 is substituted to glutamic acid
- 3) PcdNA P60E-HA; gene encoding mutant wherein poline at the 60 position of DOCK2 is substituted to glutamic acid
- 4) PcdNA F63E-HA; gene encoding mutant wherein phenylalanine at the 63 position of DOCK2 is substituted to glutamic acid

Amino acid sequence from 10-89 including DOCK2 SH3 domain is shown in Fig.3A. For each of total cell lysate, an approximately 100-KD band corresponding to ELMO1 for anti-V5 antibody was detected (Fig.3B; top). However, when targeting to immunoprecipitants using anti-HA antibody, the band corresponding to ELMO1 was not detected except for those introduced with PcdNA DOCK2-HA and PcdNA L27E-HA (Fig.3B; middle). On the other hand, when any one of the genes has been introduced, DOCK2 and DOCK2 SH3 mutant expressions were almost of the same level (Fig.3B; lower). The above results show that the association of DOCK2 and ELMO1 is completely inhibited by substituting a single amino acid of SH3 domain. Therefore, it has been clarified that DOCK2 is bound to ELMO1 via its SH3 domain.

Example 4 (Binding of C terminus domain of ELMO1 and DOCK2)

Next, to identify the functional domain of ELMO1 binding with DOCK2, gene constructs encoding various ELMO1-deleted mutants were constructed by using PcdNA V5His vector, and were analyzed by introducing these into 293T cells with PcdNA DOCK2-HA. Genes herein used, encoding ELMO1-deleted mutants are as follows, which are shown schematically in Fig.4A.

- 1) PcdNA ELMO1-del11-V5; gene encoding amino acid residues at the position 147-727 of ELMO1
- 2) PcdNA ELMO1-del18-V5; gene encoding amino acid residues at the position 345-727 of ELMO1
- 3) PcdNA ELMO1-del110-V5; gene encoding amino acid residues at the position 1-613 of ELMO1

For each of the total cell lysate, band corresponding to ELMO1 or its deleted mutant was detected with anti-V5 antibody (Fig 4B; top). However, as for immunoprecipitants with anti-HA antibody, bands reacting to anti-V5 antibody were observed when genes encoding full length ELMO1, ELMO1-del11 and ELMO1-del18 were introduced, but not when PcdNA ELMO1-del110 lacking amino acid residues at the position 614-727 of ELMO1, was expressed (Fig.4B; middle, bottom). From these, C terminus domain including amino acid residues at the position 614-727 of ELMO1 was revealed to be crucial for the association of DOCK2 SH3 domain. From these results, it has been clarified that ELMO1 was bound with DOCK2 in its C terminus domain.

Example 5 (Binding of N terminus domain of ELMO1 and Tiam1)

Tiam 1 has been identified as a molecule that determines the invasion of thymoma cell lines, and is known to function as Rac-specific GDP/GTP exchange factor (GEF) (Cell 77, 537-549, 1994; Nature 375, 338-340, 1995). As the association of DOCK2 and ELMO1 is necessary for the full activation of Rac, it has been estimated that DOCK2 might recruit Tiam1 via ELMO1. To investigate this assumption, from a Tiam1 gene amplified by PCR method from cDNA derived from mouse organs, a construct encoding Tiam1 wherein HA tag was introduced at its C terminus (PCI

Tiam1-HA) was constructed with the use of PCI vector (Promega), introduced into 293T cells with genes encoding full length or various ELMO1-deleted mutants (PcDNA ELMO1-V5, PcDNA ELMO1-delPH-V5, PcDNA ELMO1-del8-V5, PcDNA ELMO1-del1), and was then analyzed. PcDNA ELMO1-delPH-V5 is a gene encoding amino acid residues at the position 1-565 and 695-727 of ELMO1. ELMO1-deleted mutants herein used are shown schematically in Fig. 5A. For each of the total cell lysate, a band corresponding to ELMO1 or its deleted mutant was detected with anti-V5 antibody (Fig.5B; top). In immunoprecipitants with anti-HA antibody, when PcDNA ELMO1-V5 and PcDNA ELMO1-delPH-V5 were introduced, bands reacting to anti-V5 antibody were detected (Fig.5B; middle, bottom). This shows that Tiam1 binds with ELMO1. However, as for mutants lacking amino acid residues from N terminus to 146, or to 344, of ELMO1, such binding was not observed (Fig.5B; middle, bottom). From these results, it has been revealed that ELMO1 is associated with Tiam1 at its N terminus.

From the above, the following has been revealed:

- 1) DOCK2 binds to the C terminus domain of ELMO1 via SH3 domain
- 2) ELMO1 binds with Tiam1 via its N terminus domain
- 3) Rac-activating ability is significantly decreased in DOCK2 mutants that cannot bind with ELMO1.

Therefore, it has been shown that DOCK2 activates Rac by recruiting Tiam1 that functions as GEF of Rac, via ELMO1 (Fig. 6).

As autoimmune diseases and graft rejection are induced when lymphocytes invade into the target tissues, DOCK2 signaling should be the excellent target to treat or prevent these diseases or pathologic conditions. The finding of the

invention shows that interaction between molecules such as DOCK2, ELM01 and Tiam1 regulate Rac activation that is essential for cell mobility. Therefore, it can be thought that by blocking the intermolecular interaction, the invasion of lymphocytes can be inhibited. Therefore, these intermolecular interactions are anticipated to be the target of drug discovery heading to the development of method for treating or preventing autoimmune diseases or graft rejection.

Industrial Applicability

According to the present invention, it is possible to elucidate the interaction between molecules of DOCK2, and to provide a substance controlling lymphocyte migration and a method to regulate lymphocyte migration targeting DOCK2. Moreover, according to the present invention, it is possible to provide preventive or therapeutic agents of autoimmune diseases or graft rejections after implantation.